Photo-Enhanced Modification of Human Skin Elastin in Actinic Elastosis by N^{ϵ}-(Carboxymethyl)lysine, One of the Glycoxidation Products of the Maillard Reaction

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Long-term incubation of proteins with glucose leads to the formation of advanced glycation end products (AGEs), which are characterized by fluorescence, brown color, and cross-linking. Formation of AGEs *in vitro* requires oxygen and is dependent on transition metal-catalyzed oxidation of glucose or Amadori products. AGEs are thought to be involved in aging and age-enhanced diseases such as diabetic complications, atherosclerosis, dialysis-related amyloidosis, and Alzheimer's disease. Chronic exposure of the skin to sunlight induces hyperplasia of the elastic tissue in the upper dermis known as actinic elastosis. Herein we used a monoclonal anti-AGE antibody (6D12) whose epitope is N^{ϵ}-(carboxymethyl)lysine (CML), one of the glycoxidation products of AGEs, and demonstrated that the lesions of actinic elastosis were modified by CML. Further immunohistochemical and immunoelectron microscopic examination with 6D12 demonstrated CML accumulates predominantly in elastic fibers especially in the amorphous electron-dense materials corresponding to photo-induced degenerated area rather than the electron-lucent region. Immunochemical analyses with enzyme-linked immunosorbent assay (ELISA) of elastase-soluble fractions demonstrated that the CML levels of the sun-exposed area were significantly higher than those of the sun-unexposed area. We conclude that ultraviolet-induced oxidation may accelerate CML formation in actinic elastosis of photoaged skin. *Key words: photoaging/advanced glycation end products. J Invest Dermatol 108:797–802, 1997*

Long-term incubation of proteins with glucose *in vitro* leads, through early-stage products such as a Schiff base and Amadori rearrangement products, to the formation of advanced glycation end products (AGEs), which are characterized by fluorescence, brown color, and cross-linking (Maillard, 1912). We prepared an anti-AGE monoclonal antibody (6D12) in mice (Horiuchi *et al*, 1991). Immunologic studies using 6D12 not only demonstrated the presence of AGEs in several human tissues but also suggested a potential link of AGEs to aging (Araki *et al*, 1992; Kimura *et al*, 1995, 1996) and age-enhanced diseases such as diabetic complications (Yamada *et al*, 1994; Makino *et al*, 1995), atherosclerosis (Kume *et al*, 1995; Horiuchi, 1996; Meng *et al*, 1996), and dialysis-related amyloidosis (Miyata *et al*, 1993, 1996). Immunologic studies using anti-AGE polyclonal antibodies were also performed in other laboratories, demonstrating the presence of AGEs in human plasma (Makita *et al*, 1992a; Bucala *et al*, 1993), hemoglobin (Makita *et al*, 1992b), atherosclerotic coronary artery (Nakamura *et al*, 1993), and lesions of Alzheimer's disease (Smith *et al*, 1994; Vitek *et al*, 1994).

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Abbreviations: AGE(s), advanced glycation end product(s); CML, N^{ϵ}-(carboxymethyl)lysine.

Several AGE structures have been identified including pyrraline (Hayase *et al*, 1989), pentosidine (Sell *et al*, 1989), N^{ϵ}-(carboxymethyl)lysine (CML) (Ahmed *et al*, 1986), and crosslines (Nakamura *et al*, 1992). It is still not known, however, whether one of these compounds contributes, as a major AGE structure, to the pathogenesis of these diseases or whether other structure(s) may be involved in this process. Although the presence of AGEs in several human tissues was successfully demonstrated by our anti-AGE monoclonal antibody (6D12) as described above, no information was available until quite recently about its AGE structure. Our recent study revealed that 6D12 is able to recognize a CML-protein adduct as an epitope (Ikeda *et al*, 1996), strongly suggesting an *in vivo* importance of CML among AGE structures.

In the skin after chronic exposure to sunlight, especially in persons with a fair complexion, hyperplasia of the elastic tissue is usually evident in the upper dermis by the age of 30. These changes found in the photoaged skin are called actinic elastosis (Kligman, 1969). Accumulated elastotic materials are thought to be derived from elastic fibers because of their reactivity with the anti-elastin antibody and sensitivity to elastase digestion (Pieraggi, 1988). Although it has not been clear how chronic solar exposure can induce accumulation of elastic fibers, deleterious effects of sunlight could often be attributed to oxidation by free radical intermediates (Sugiyama *et al*, 1984; Buettner *et al*, 1987; Miyachi, 1993; Pentland, 1994). It is therefore possible that ultraviolet-induced oxida-

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tive stress may have some role in the pathogenesis of actinic elastosis through the formation of oxidation products.

CML is formed *in vitro* by oxidative cleavage of Amadori adducts (Ahmed *et al*, 1986; Dunn *et al*, 1991) or Schiff bases (Glomb and Monnier, 1995) or by modification with glyoxal generated directly through autoxidation of glucose (Wells-Knecht *et al*, 1995) and has been proposed as a potential biomarker of oxidative damage of tissue proteins *in vivo* (Baynes, 1991; Fu *et al*, 1994). CML is therefore termed a "glycoxidation product" among AGE structures. There is currently no evidence that AGEs result from UV-induced oxidation. To test this hypothesis, we have attempted to determine whether CML modification could occur in the lesions of actinic elastosis. Immunologic studies using 6D12, a monoclonal antibody specific for CML structure, demonstrated CML accumulation predominantly in elastic fibers especially in the amorphous electron-dense material corresponding to photo-induced degenerated area. These results indicate that ultraviolet-induced oxidation may accelerate CML formation in actinic elastosis of the photoaged skin.

MATERIALS AND METHODS

Tissue Preparation Fifty-seven specimens were obtained from 51 Japanese patients undergoing dermatologic surgery for the treatment of benign and malignant epithelial tumors at Kumamoto University School of Medicine Hospital **(Table I)**. Specimens were obtained from various sites including face (cheek, forehead, and nose), neck, back of the hand, forearm, shoulder, chest, abdomen, back, groin, and sole of both male and female patients. Their ages ranged from 9 to 100 y. The peripheral tissues surrounding the tumor were procured at the time of surgery. Prior informed consent was obtained in all cases. When the subjects were juveniles, informed consent was obtained from their parents. Specimens utilized in the study were all histologically tumor-free and contained epidermis and underlying dermis. The samples were sectioned and processed in two ways: one was formalin-fixed and paraffin-embedded and the other was rapidly frozen and stored at −80°C. Paraffin-embedded samples in 6-μm sections were stained with hematoxylin and eosin for diagnosis and the frozen sections were subjected to immunohistopathologic studies. The specimens obtained from patients 48, 49, and 51 were processed for immunoelectron microscopy.

Antibodies Anti-AGE monoclonal antibody (6D12) was produced by immunization of BALB/c mice with AGEs coupled to bovine serum albumin (BSA) as described (Horiuchi *et al*, 1991). A recent immunochemical study demonstrated that 6D12 recognizes one of the AGE structures, N^{ϵ}-(carboxymethyl)lysine (Ikeda *et al*, 1996). 6D12 was used for the current study except that a rabbit polyclonal anti-CML antibody was used for confocal laser scanning microscopy. This polyclonal antibody (CML-PA) was shown to recognize CML as its epitope (Ikeda *et al*, 1996). To identify elastin, anti-elastin monoclonal antibody (Elastin Products, Owensville, MO) was used.

Immunohistochemical Studies The rapidly frozen samples were stained as described (Kume *et al*, 1995) with the anti-elastin antibody and 6D12. The rapidly frozen samples were sectioned into 6 μm, air-dried, and fixed in 100% ice-cold acetone for 10 min. Endogenous peroxidase was inactivated by incubating the sections with 0.3% hydrogen peroxide in methanol for 30 min. These sections were washed three times with phosphate-buffered saline (PBS) and incubated overnight at 4°C with the primary antibody, 6D12 (0.5 μg per ml), or the anti-elastin antibody (1:4000 dilution). After washing three times with PBS, the sections were incubated at room temperature for 30 min with biotinylated horse anti-mouse IgG antibody (Vector Laboratories, Burlingame, CA). The immunoreaction was detected with the avidin-biotin-horseradish peroxidase complex (Vector Laboratories, Burlingame, CA), using 4-dimethylaminoazobenzene as a chromogen. Nonimmune mouse IgG was used as negative control. To evaluate the specific immunoreactivity of 6D12 to CML, the tissue specimens were immunostained with 6D12 (1 μg per ml) in the presence of 100 μg CML-BSA per ml. CML-BSA was prepared by incubating BSA with glyoxylic acid and $NaCNBH_3$ as described (Ikeda *et al*, 1996).

Confocal Laser Scanning Microscopy For confocal laser scanning microscopy, double labeling was also performed. The rapid frozen samples were sectioned into 10-μm sections and air-dried. The sections were incubated simultaneously with a rabbit anti-CML polyclonal antibody (1.0 μg per ml) and the anti-elastin antibody (1:4000 dilution) at 4°C overnight. After washing with PBS, sections were incubated at room temperature for 1 h with fluorescein isothiocyanate-conjugated anti-rabbit IgG at a titer of 1:50 (green; Serotec, Oxford, U.K.) and then with rhodamine-conjugated anti-mouse IgG (1:50 dilution) (red; Leinco Technologies, Ballwin, MO). One of these sections was used to observe under a confocal laser scanning microscope (MRC-1024; BioRad, Hemel Hempstead, U.K.). A yellow fluorescence occurs when both green and red stains bind to the same location. Nonimmune rabbit and mouse serum (Cappel, West Chester, PA) were used as negative control. No immunoreaction was observed in formalin-fixed and paraffin-embedded specimens.

Immunoelectron Microscopy The specimens from the face of patients 48, 49, and 51 were subjected to immunoelectron microscopic study by a post-embedding method using anti-elastin antibody and 6D12. The tissue samples were fixed with Karnovsky fixative for 24 h at 4°C, washed with PBS, dehydrated in a graded series of ethanol, and embedded in LR White resin (London Resin, Berkshire, U.K.). Ultrathin sections were floated on drops of buffer A (0.5% BSA, 0.1% gelatin, and 20 mM NaN_3 in PBS) for 15 min and incubated for 1 h at room temperature with either the anti-elastin antibody (1:500 dilution) or 6D12 (5 μg per ml). The sections were washed with buffer and further incubated for 30 min with 15-nm colloidal gold-conjugated goat anti-mouse IgG antibody (Aurion, Wageningen, Netherlands) in buffer A. These sections were washed with buffer A, PBS, and distilled water, stained with uranyl acetate, and observed under a Hitachi H300 electron microscope. For double labeling with different size of gold particles, 6- and 15-nm colloidal gold-conjugated goat anti-mouse IgG antibodies were used to localize the bound anti-elastin antibody and 6D12, respectively.

Biochemical Analysis We studied skin tissues obtained from skin surgery of sun-exposed and sun-unexposed area in 18 individuals. Elastin was solubilized with elastase (Fleischmajer and Lara, 1966), and its immunoreactivity to 6D12 was determined by noncompetitive enzyme-linked immunosorbent assay (ELISA) using the following procedure (Horiuchi *et al*, 1991). Each skin specimen was rapidly frozen and sliced into 10-μm sections. Skin sections were washed three times with PBS and incubated overnight with elastase (Wako Chemical, Osaka, Japan) in 0.1 M Tris(hydroxymethyl)aminomethane hydrochloride buffer (pH 8.8) at 37°C. The elastase-soluble fraction was collected as "peptide fraction." Protein concentrations were determined by bicinchoninic acid protein assay reagent (Pierce, Rockford, IL) using BSA as a standard. Immunochemical assays were performed at room temperature in noncompetitive ELISA. Each well of a 96-well microtiter plate was incubated for 60 min with 5 μg of the peptide fraction in 50 mM sodium carbonate buffer (pH 9.6). Wells were washed three times with PBS containing 0.05% Tween 20 (buffer B), blocked with 0.5% gelatin, washed with buffer A, and reacted for 60 min with 0.1 ml of buffer B containing 1.0 μg 6D12 per ml. The wells were then washed with buffer B and reacted with horseradish peroxidase-conjugated anti-mouse IgG, followed by reaction with 1,2-phenylenediamine dihydrochloride. The reaction was terminated by 1.0 M sulfuric acid, and the absorbance at 492 nm was read on a micro-ELISA plate reader. The immunoreactivity in the presence of 100 μg CML-BSA per ml was defined as the nonspecific binding level. The specific CML level was determined by subtracting the level of nonspecific binding from the total binding level obtained in the absence of CML-BSA and expressed as the absorbance at 492 nm. In five cases (cases 46, 47, and 49–51), skin samples were obtained both from sun-exposed and sun-unexposed areas in the same individual. The results were analyzed by matched paired t test. The CML levels were also determined in seven sun-exposed skin specimens (cases 1–3, 5, 6, 17, and 18), and another six sun-unexposed ones (cases 38–40, 42, 43, and 45). For immunochemical experiments using ELISA, the same set of experiment was repeated at least three times and the representative data were shown in each figure. The results were analyzed by the group comparison using the Aspin–Welch t test.

RESULTS

Accumulation of CML in Elastic Fibers in the Photoaged Skin Fifty-seven specimens obtained from the sun-exposed or sun-unexposed skin were stained with hematoxylin and eosin. **Figure 1*a*** shows the typical elastic-tissue changes commonly observed in the sun-exposed skin samples in which massive quantities of thickened, tangled, degraded elastotic material was present in the upper dermis of the photoaged skin, as reported (Kligman, 1969). To examine the presence of elastin in these degraded elastotic materials, sections were immunostained with anti-elastin antibody. These materials were stained by anti-elastin antibody **(Fig 1*b*)**, confirming that elastin is a major component (Pieraggi *et al*, 1988). Upon serial sections, these elastotic materials were also strongly stained with 6D12 **(Fig 1*c,d*)**, and this immunoreaction was significantly inhibited by an excess of CML-BSA **(Fig 1*e*)** but

Table I. Clinical Data, Degree of Actinic Elastosis, Grade of Elastic-Tissue Changes, and CML Accumulation in 51 Subjects[a]

Case	Age (y)	Sex	Region	Elastosis[b]	Grade[c]	CML[d]
1	100	F[e]	Face	U & L, B	4+	+
2	94	F	Face	U & L, B	4+	+
3	92	F	Face	U & L, B	4+	+
4	90	M	Face	U & L, B	4+	+
5	90	M	Face	U & L, B	4+	+
6	88	F	Face	U & L, B	4+	+
7	86	M	Face	U & L, B	4+	+
8	85	F	Face	U & L, B	4+	+
9	84	M	Face	U & L, B	4+	+
10	80	F	Face	U & L, B	4+	+
11	80	M	Face	U, B	3+	+
12	79	F	Face	U & L, B	4+	+
13	78	M	Face	U, P	4+	+
14	76	M	Face	U & L, B	4+	+
15	75	F	Face	U & L, B	4+	+
17	71	F	Face	U & L, B	4+	+
18	67	F	Face	U & L, B	4+	+
19	66	M	Face	U, P	4+	+
20	66	M	Face	U & L, P	4+	+
21	53	M	Face	U & L, P	3+	+
22	36	M	Face	NV	0	+L
23	32	M	Face	NV	1+	+L
24	31	F	Face	NV	1+	+L
25	28	M	Face	NV	0	+L
26	14	F	Face	NV	0	−
27	10	F	Face	NV	0	−
28	81	F	Neck	U & L, B	4+	+
29	42	F	Neck	NV	1+	+L
30	19	F	Neck	NV	1+	−
31	14	M	Neck	NV	0	−
32	22	F	Back of the hand	NV	0	−
33	84	F	Fore arm	NV	1+	−
34	38	M	Fore arm	NV	1+	−
35	34	F	Fore arm	NV	0	−
36	45	M	Shoulder	NV	0	−
37	17	F	Shoulder	NV	0	−
38	25	M	Chest	NV	0	−
39	64	F	Abdomen	NV	0	−
40	61	F	Back	NV	0	−
41	9	M	Back	NV	0	−
42	88	M	Groin	NV	0	−
43	15	F	Groin	NV	0	−
44	32	F	Sole	NV	0	−
45	11	F	Sole	NV	0	−
46	91	F	Face	U & L, B	4+	+
	91	F	Chest	NV	0	−
47	88	F	Face	U & L, B	4+	+
	88	F	Chest	NV	0	−
48	88	F	Face	U & L, B	3+	+
	88	F	Chest	NV	0	+L
49	85	F	Face	U & L, B	4+	+
	85	F	Chest	NV	0	−
50	83	F	Face	U & L, B	4+	+
	83	F	Chest	NV	0	+L
51	72	M	Face	U, P	3+	+
	72	M	Chest	NV	0	−

[a] Fifty-seven specimens of skin surgery from 51 Japanese ranging in age from 9 to 100 y were examined histologically by hematoxylin and eosin staining and immunohistochemically with anti-elastin antibody and anti-AGE antibody (6D12).

[b] Lesions of actinic elastosis were examined by hematoxylin and eosin staining and classified into two groups: a band-like type (B) in which one elastotic material was continuous with another exhibiting a band-like structure and a "patchy" type (P) in which the distribution of the elastotic material was focal or patchy. These were found in the upper (U) and/or lower (L) dermis. NV, none visualized.

[c] Elastic tissue changes were examined by hematoxylin and eosin staining and an immunohistochemical study with anti-elastin antibody. Tissue changes were scored according to Kligman (Kligman, 1969) as follows: 0, no change; 1+, simple increase without thickening; 2+, greater hyperplasia with thickening and curling; 3+, marked hyperplasia, thickening, curling, and frequent branching; 4+, almost complete replacement of the dermis by a dense tangle of thickened, disorderly fibers accompanied by disorganization into murky amorphous masses.

[d] CML accumulations determined by immunohistochemical studies with 6D12 were scored as follows: +, strongly positive corresponding to elastic tissue changes; +L, linearly positive corresponding to fine elastic fibers; −, negative.

[e] F, female; M, male.

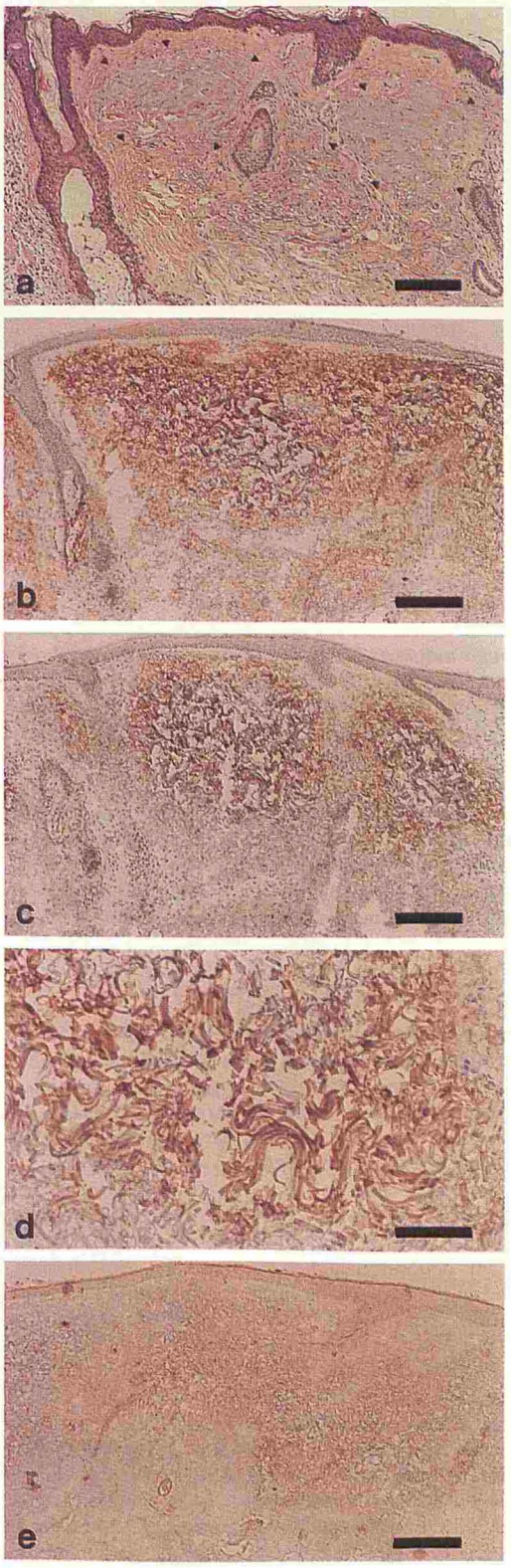

Figure 1. Accumulation of CML in elastic fibers in the photoaged skin. Separate sections of the same specimen obtained from the face of a 91-y-old female (case 46) were used for immunohistochemical studies. *(a)* Human actinic elastosis (hematoxylin and eosin-stained). Tangled masses of basophilic elastotic material (➤) fill the upper dermis. *(b)* Immunologic detection of elastic fibers in the corresponding region. The rapidly frozen samples were stained with anti-elastin monoclonal antibody. *(c)* Immunoreaction with anti-AGE monoclonal antibody (6D12). *(d)* Higher magnification of *c*. *(e)* Immunoreaction with 6D12 was completely inhibited by excess amounts of CML-BSA (100 μg per ml). *Scale bars*, *(a–c,e)* 200 μm; *(d)* 100 μm.

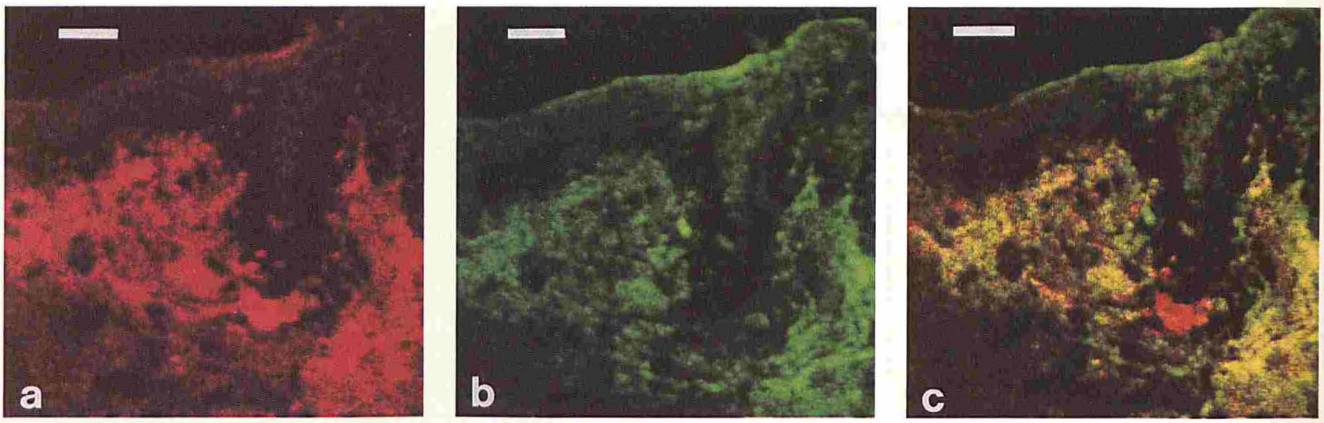

Figure 2. Co-localization of CML and elastin in the photoaged skin. The specimen is obtained from the face of a 91-y-old female (case 46). The rapidly frozen section was incubated in the mixture of rabbit anti-CML polyclonal antibody and mouse anti-elastin monoclonal antibody, followed by incubation with fluorescein isothiocyanate-conjugated anti-rabbit IgG (green) and then with rhodamine-conjugated anti-mouse IgG (red). Confocal laser scanning microscopy reveals that the abnormal elastotic material stained red *(a)* is co-localized with CML stained green *(b)*. Co-localization of elastin and CML was demonstrated by uniform yellow staining *(c)*. *Scale bars*, 20 μm.

not with BSA (data not shown). These lesions were not stained by nonimmune mouse IgG used as a control (data not shown). These results indicated that the immunoreaction is specific for CML and that the elastic fibers probably undergo CML modification *in vivo*. The latter argument is based on a similar staining pattern with 6D12 **(Fig 1*c*)** and anti-elastin antibody **(Fig 1*b*)** and the presence of 6D12-positive material on elastic fibers **(Fig 1*d*)**. Further immunologic study using a double-labeling method with anti-elastin antibody and anti-CML antibody was performed to examine the co-localization of elastin and CML. The single section obtained from the face of a 91-y-old female (case 46) was double-labeled and observed with a confocal laser scanning microscope. Red and green color demonstrated the immunoreaction of elastin and CML, respectively **(Fig 2*a,b*)**. Their co-localization was demonstrated by yellow color in the same section **(Fig 2*c*)**.

Immunoreactivity of 6D12 with Sun-Exposed Skin but Not with Sun-Unexposed Skin As shown in **Table I**, 28 of 57 skin specimens were obtained from sun-exposed areas, including 27 from the face (cases 1–21 and 46–51) and one from the neck (case 28) of individuals older than 53 y. All specimens showed actinic elastosis with marked elastic-tissue changes (grade 3 or 4 by Kligman's classification). The elastotic materials from all these specimens reacted with 6D12. In contrast, 14 skin specimens from regions other than the face or neck (cases 32–45) without significant actinic elastosis did not exhibit CML deposition, indicating that CML deposition is likely to correspond to the hyperplasia of the elastic tissues in actinic elastosis. Furthermore, comparison between exposed and unexposed areas from the same individual in 6 cases more than 72 y of age (cases 46–51) showed that CML accumulation was again restricted to actinic elastosis of sun-exposed skin. These immunohistochemical data therefore suggest that CML modification occurs in actinic elastosis in quite a specific manner. In addition to these clear and typical cases, weak but significant CML deposition was also detected in fine elastic fibers of sun-exposed areas from 5 cases (cases 22–25 and 29) less than 42 y of age. These lesions, although without a typical pathologic finding of actinic elastosis by hematoxylin and eosin staining, could reflect the early phase of actinic elastosis.

Accumulation of CML in the Electron-Dense Material We also examined the ultrastructure of 6D12-reactive material in hyperplastic elastic fibers in an immunoelectron microscopic study. The skin specimen from the face of a 88-y-old female (case 48) was reacted with anti-elastin antibody **(Fig 3*a,b*)** and 6D12 **(Fig 3*c,d*)** followed by visualization with colloidal gold-conjugated anti-mouse IgG antibody. The immunolabeling for elastin and CML was present in the elastotic degenerated fibers **(Fig 3*a,c*)**, whereas the immunoreactivity of collagen fibers to 6D12 was negligible. Although the gold particles on the elastic fibers were nearly invisible in **Fig 3*a*** and ***c***, higher magnification clearly showed that the immunolabeling for elastin was restricted to the amorphous electron-lucent material corresponding to elastin **(Fig 3*b*)**, whereas immunolabeling for CML was present in the amorphous electron-dense material of elastotic degenerated fiber **(Fig 3*d*)**. Elastin and CML were further examined in the same sample by a double-labeling method with small and large gold particles, respectively. By double labeling, the immunoreactivity to CML occurred selectively in the amorphous electron-dense material, whereas that to elastin was restricted to the electron-lucent region in the elastotic degenerated fiber **(Fig 3*e*)**. Since this electron-dense material is known to appear in elastic fibers in relation to photoaging (Banfield *et al*, 1963), it is likely that CML accumulation in this area reflects an photoaging-related alteration.

Immunochemical Quantification of CML Levels in Skin Elastin To further confirm the presence of CML in elastic fibers, skin samples were solubilized with elastase and the amount of CML in their elastase-soluble fraction was determined with an ELISA. The CML level of the sun-exposed area was significantly higher than that of the unexposed areas in the same individual ($n = 5$, $p = 0.0139$; **Fig 4*a***). Moreover, the mean CML level in the sun-exposed skin samples ($n = 7$) was significantly higher than that of unexposed skins ($n = 6$, $p = 0.0187$; **Fig 4*b***). These immunochemical studies of elastase-soluble fraction of the skin also support the selective accumulation of CML in elastic fibers.

DISCUSSION

This study shows that the elastin in the photoaged skin of "actinic elastosis" is modified by CML, one of the glycoxidation products among AGEs. By using a monoclonal antibody specific for CML (6D12), we evaluated the CML accumulation in the sun-exposed or sun-unexposed skins obtained from 51 human subjects. Immunohistochemical and immunoelectron microscopic examinations demonstrated CML accumulation predominantly in elastic fibers especially corresponding to photo-induced degenerated area. CML is formed *in vitro* by oxidative cleavage of Amadori adducts (Ahmed *et al*, 1986; Dunn *et al*, 1991) or Schiff bases (Glomb and Monnier, 1995) or by modification with glyoxal generated directly through autoxidation of glucose (Wells-Knecht *et al*, 1995). Because of the interplay between glycation and oxidation in its formation, CML is considered to be a glycoxidation product and a potential biomarker of carbohydrate-dependent oxidative damage of tissue proteins *in vivo* (Baynes, 1991). At the same time, a recent study has suggested

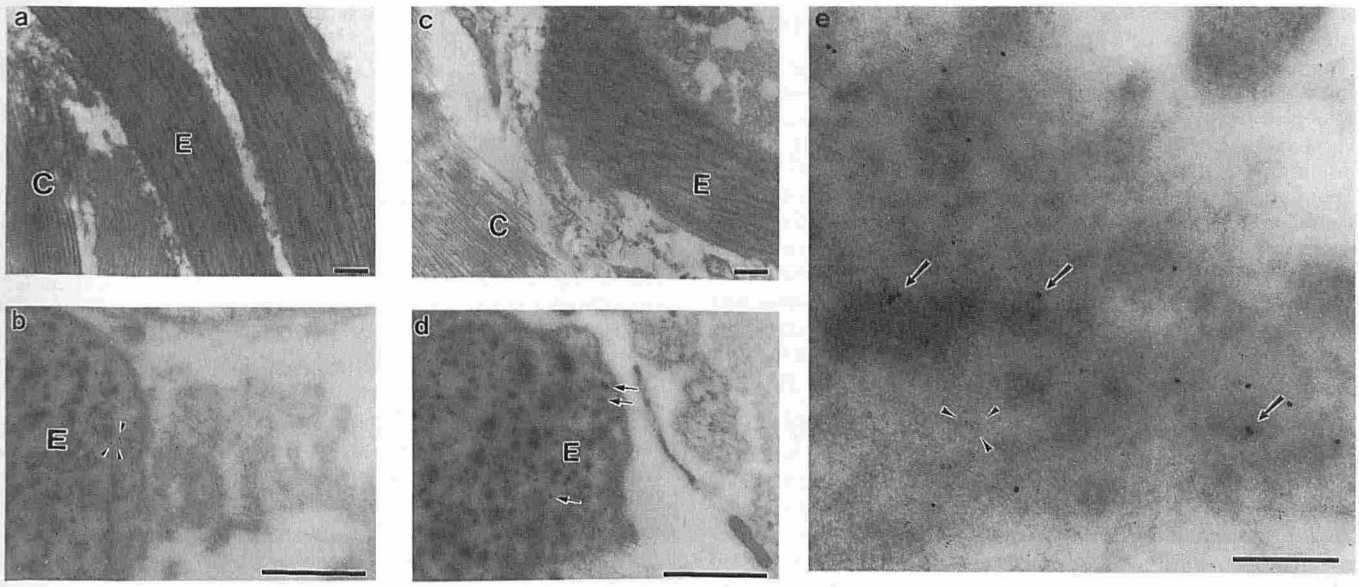

Figure 3. Accumulation of CML in the electron-dense material. The skin specimen from the face of a 88-y-old female (case 48) was subjected to immunoelectron microscopical study using anti-elastin antibody and 6D12, followed by colloidal gold-conjugated goat anti-mouse IgG antibody. *(a)* Immunoelectron microscopical localization of elastin with anti-elastin antibody. Note the selective accumulation of gold particles in longitudinal section of the elastotic degenerated fibers (E) but rarely in collagen fibers (C). *(b)* Higher magnification of an elastotic degenerated fiber in cross-section immunostained with anti-elastin antibody. Inside an elastic fiber (E), the gold particles are located in the amorphous electron-lucent regions (➤). *(c)* Immunoelectron microscopical localization of CML with 6D12. The gold particles conjugated with 6D12 are found selectively in longitudinal section of the elastotic degenerated fibers (E) but not in collagen fibers (C). *(d)* Higher magnification of an elastotic degenerated fiber in cross-section immunized with 6D12, showing the selective accumulation of the gold particles in the amorphous electron-dense regions (→). *Scale bars*, 1.0 μm. *(e)* Double labeling using anti-elastin antibody and 6D12 in the elastotic degenerated fiber. Note that CML indicated by larger gold particles (→) are selectively present in the electron-dense material, whereas elastin indicated by smaller particles (➤) are present in the electron-lucent region. *Scale bar*, 0.5 μm.

that CML is also important as a lipid peroxidation product, because it was generated from arachidonic acid without glucose (Fu *et al*, 1996). Previous chemical analyses of acid hydrolysates of tissue samples quantitated the amount of CML in human lens, skin, and urine and its increase with aging and in diabetes (Dunn *et al*, 1989; Dunn *et al*, 1991; Knecht *et al*, 1991). Consistent with these observations, our recent immunologic studies using 6D12 also demonstrated an increase in CML modification with age in human lens (Araki *et al*, 1992) and brain (Kimura *et al*, 1995, 1996) and at an accelerated rate under disease states such as diabetic complications (Yamada *et al*, 1994; Makino *et al*, 1995), atherosclerosis (Kume *et al*, 1995; Meng *et al*, 1996), and dialysis-related amyloidosis (Miyata *et al*, 1993; Miyata *et al*, 1996). It is likely, therefore, that CML formation *in vivo* does directly reflect oxidative stress *in situ* and that the amounts of its formation are increased significantly under oxidation-enhanced pathologic conditions including actinic elastosis of photoaged skin.

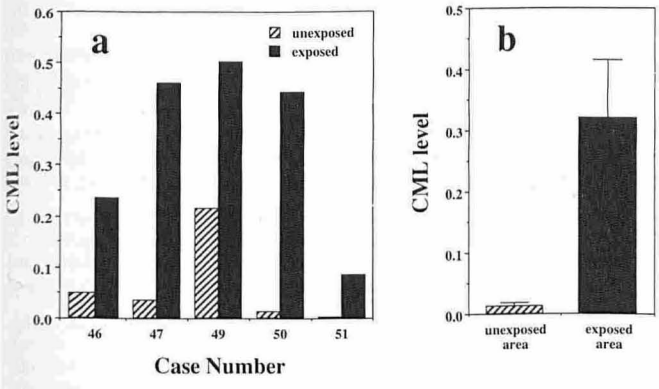

Figure 4. Immunochemical quantification of CML levels in skin elastin. Elastin was solubilized with elastase and its immunoreactivity to anti-AGE monoclonal antibody (6D12) was examined by noncompetitive ELISA and was expressed as the absorbance at 492 nm. The specific CML level was determined by subtracting the level of nonspecific binding in the presence of 100 μg CML-BSA per ml from the total binding level obtained in the absence of CML-BSA. *(a)* The CML level in the sun-exposed skin area was significantly higher than that of the sun-unexposed area of the same individuals in five cases (cases 46, 47, and 49–51, $p = 0.0139$). *(b)* The mean value of CML levels of the sun-exposed skin from seven cases (cases 1–3, 5, 6, 17, and 18) was significantly ($p = 0.0187$) higher than that of the sun-unexposed skin from six cases (cases 38–40, 42, 43, and 45). *Error bars*, SEM (exposed, $n = 7$; unexposed, $n = 6$).

Our study was able to demonstrate the presence of CML in elastic fibers but not in collagen fibers. Previous studies using gas chromatography-mass spectrometry, however, demonstrated CML in human skin collagen and the significant increase in its amount with age (Dunn *et al*, 1991; Dyer *et al*, 1993). The precise reason for this inconsistency is not clear, but it could be explained in part by two considerations. One is a difference in the sensitivity between the chemical and immunologic methods used for measurement of CML. Although immunohistochemical study can demonstrate the distribution and accumulation of the product, it does not always reflect its absolute amount in tissue. The other explanation is a difference in the specimens used in these studies; the photoaged skin of actinic elastosis was examined in the current study, whereas healthy subjects were used in the previous ones.

There is another related issue in our study between immunohistochemical results **(Table I)** and immunochemical results from an ELISA **(Fig 4)**. The immunoreactivity of the sun-unexposed skin of case 49 is significantly higher than that of the sun-exposed skin of case 51 in an ELISA, whereas no immunostaining was observed in the sun-unexposed skin of case 49. The results obtained by noncompetitive ELISA do not always reflect the absolute amount of CML in the skin *in vivo*, because it is difficult to equally solubilize skin tissue and equally coat the elastase-soluble fraction to the microtiter plate in a noncompetitive ELISA. Further studies are

needed to determine the absolute amount of CML by analytical examinations such as mass spectrometry.

There is a possibility that AGEs may be formed through chronologic aging and diabetes. The immunohistochemical and immunochemical studies performed on paired samples, without the confounding factors such as age and serum glucose, however, suggest that CML modification is directly derived from chronic sun exposure.

Immunoelectron microscopic study identified the site of CML accumulation in amorphous electron-dense materials of elastotic degenerated fiber. Since these electron-dense materials are known to appear in elastic fibers in relation to photoaging (Banfield *et al*, 1963), it is highly likely that the site of CML accumulation in actinic elastosis is the photo-induced degenerated area. It is possible, however, that several other matrix components including microfibrillar antigen (Chen *et al*, 1986) or fibrillin (Bernstein *et al*, 1994) undergo modification with CML.

AGE structures other than CML such as pyrraline, pentosidine, and cross-lines are known. In a preliminary experiment, the lesions of actinic elastosis were stained by our polyclonal anti-AGE antibody whose epitope is not known but differs from CML, pentosidine, or cross-lines. In addition, these lesions were also stained by the anti-pentosidine polyclonal antibody, indicating the presence of pentosidine and other AGE structure(s) in these lesions. It is evident therefore that UV-induced oxidation (Sugiyama *et al*, 1984; Buettner *et al*, 1987; Miyachi, 1993; Pentland, 1994) plays a crucial role in AGE modification of elastic fibers of actinic elastosis with CML, pentosidine, and other oxidation-required AGE structure(s). Thus, although it is not clear whether AGE accumulation in the skin *per se* is a cause of the disease or simply its effect, it could at least serve as a biomarker for the duration and severity of oxidative damage to the skin *in vivo*. To support this idea, further studies are needed to elucidate direct effects of UV irradiation on the formation of AGEs *in vitro* and *in vivo*.

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